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PHYSIOLOGICAL ECOLOGY - ORIGINAL RESEARCH

The confounding effects of source isotopic heterogeneity on consumer–diet and tissue–tissue stable isotope relationships

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Abstract Stable isotope analysis of consumer tissues document patterns of resource use because data are linearly related to isotope compositions of their source(s) (i.e., food, water, etc.). Deviations in parameters estimated for these relationships can arise from variations in consumer tissue–diet spacing (Δ_{TS}) and the level of isotopic heterogeneity in the source(s). We present a set of simple hypotheses that distinguish between the effects of Δ_{TS} and source isotope heterogeneity. The latter may arise via mixed diets, during tissue turnover, or by isotopic routing of dietary components. We apply these concepts to stable carbon and nitrogen isotope relationships between gut contents and body tissues of large mammal herbivores from mixed C_3/C_4 South African savannas and test predictions based on the compound- and/or time-specific data archived within each material. Predicted effects of source isotope heterogeneity are readily detected in carbon isotope relationships between materials representing different time periods or comprising bulk versus protein-only diet components. Differences in Δ_{TS} of carbon isotopes across mammal herbivore species with very different feeding niches (and diet isotope compositions) are likely to be small or non-existent in these habitats. Variations in Δ_{TS} estimated for nitrogen isotopes are much greater, leading to inconsistencies that cannot be explained by diet or trophic level effects alone. The effects of source heterogeneity on isotopic relationships generate numerical artefacts that have been misinterpreted as variations in Δ_{TS}. We caution against generalized application of hypotheses based on assumptions of source isotopic homogeneity, even for single diets commonly used in laboratory studies. More careful consideration of how heterogeneity affects consumer–diet relationships is needed for many field and laboratory systems.

Keywords Bone collagen · Carbon isotopes · Fractionation · Gut contents · Hair · Nitrogen isotopes · Turnover

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Introduction

Stable isotope analysis of consumer body tissues and excreta is a widely applied method to reconstruct ecological and life-history parameters of wildlife, such as nutrient

acquisition, resource allocation, trophic relationships, ecological niche separation, community assembly, and migration (Hobson 1999; Hobson et al. 2004; Layman et al. 2007; Newsome et al. 2007). The approach is based on the principle "you are what you eat", an expression of the generally linear relationship between the isotope compositions of consumer tissues and their diets (DeNiro 1978, 1981). An important extension of this principle is that analyses of different consumer tissues are informative about changes in diet sources. Each tissue captures information specific to its growth and metabolic rate, so that isotopic comparisons between them resolve diets over a variety of time frames and scales (Tieszen et al. 1983; Hobson 1999; Phillips and Eldridge 2006; Bauchinger and McWilliams 2009). Further, differences in the biochemical composition of tissues means they will often reflect the isotopic composition of different diet components, such as proteins, lipids, or bulk diets; consequently, the comparison of multiple tissues provides insights into how nutrients are allocated (Ambrose and Norr 1993; Hobson et al. 2004). In general, however, the information needed to answer such questions, including reliable empirical estimates of isotope turnover and incorporation rates into specific tissues, and of how dietary constituents are routed across them, is scarce (Martínez del Rio et al. 2009). In many cases, interpreting isotope relationships between multiple tissues is difficult, or must be made a posteriori.

A formal understanding of isotopic relationships amongst consumer tissues requires, as a first step, a general understanding of the relationship between consumer tissues and diet sources. This relationship makes it possible to trace the dietary source(s) of a consumer's tissue and, in addition, regression parameters derived from known (experimental) datasets are instructive about systems where diet isotope compositions are unknown, such as studies of free-ranging wildlife (Felicetti et al. 2003; Caut et al. 2009, 2010; Robbins et al. 2010). Recent studies have drawn attention to variations in the parameter estimates for such regressions and the implications of these variations for successful application of the data (Caut et al. 2010; Robbins et al. 2010). However, these studies provide different interpretations of the factors that influence the regressions, which suggests that at least one of the factors must be misleading. A generalized concept of the meaning of parameters of consumer–diet isotopic relationships is thus warranted. Our aim here is to present a simplified generalization of consumer–diet relationships and its relevance to relationships between tissues. We test predictions for the latter using a dataset of stable carbon and nitrogen isotope ratios in multiple body tissues and gut contents of free-ranging mammal herbivores from South African savannas.

Relationships between consumer and diet isotope ratios

Here, we simulate a set of simple consumer–diet isotopic relationships and the regression parameters associated with each scenario. The simulations are based on a hypothetical group of 40 individuals, divided into two species (j and k). Each individual's tissue is derived from resource S, with isotope composition δS. Values for δS for each individual are drawn randomly from a normal distribution, with a true mean of 0 and standard deviation (SD) of 1. In the simplest scenario, the isotope composition of the consumer's tissue (δT) is a linear function of δS:

$$\delta T = \Delta_{TS} + \delta S \quad (1)$$

The intercept of Eq. 1, Δ_{TS}, is the isotopic spacing between δT and δS (i.e., $\delta T - \delta S$; but see Materials and methods, and Auerswald et al. 2010 for a critique). In animal diet studies, this parameter is also referred to as the consumer–diet fractionation, but other terms, such as discrimination, enrichment factor, and trophic enrichment factor are commonly, though not consistently, used (Cerling and Harris 1999; Caut et al. 2008; Martínez del Rio et al. 2009; Auerswald et al. 2010). Regardless of terminology, most authors agree that Δ_{TS} presents the most challenging constraint to stable isotope applications in ecology, and is the subject of much debate (including the present study).

Equation 1 also has the property of being a 1:1 relationship (slope = 1.0; Fig. 1a); simply, each individual "is what it eats" plus Δ_{TS}. In most experimental datasets, however, the slope of the δT (δS) function is significantly smaller than 1.0 (Hilderbrand et al. 1996; Caut et al. 2010; Robbins et al. 2010; Codron et al. 2011). It has been hypothesized that this happens because of variations in Δ_{TS} (Caut et al. 2010; Robbins et al. 2010). Variations in Δ_{TS} within and across systems are well-known and have been attributed to a variety of behavioral, physiological, and analytical factors (Bearhop et al. 2002; Caut et al. 2008, 2009; Martínez del Rio et al. 2009; Newsome et al. 2010; Robbins et al. 2010). For example, physiological effects that influence food assimilation may differ between species and thus lead to differences in Δ_{TS} across taxa (Passey et al. 2005). We simulate this scenario by rewriting Eq. 1 for species-specific Δ_{TS} values, $\Delta_{TS,j}$ and $\Delta_{TS,k}$:

$$\delta T_j = \Delta_{TS,j} + \delta S$$

and

$$\delta T_k = \Delta_{TS,k} + \delta S \quad (2)$$

In this instance, the slope of the δT (δS) function remains close to 1.0, but variation around the regression increases (r^2 declines) because there are two intercepts (Fig. 1b). Thus, one should be able to readily detect dif-

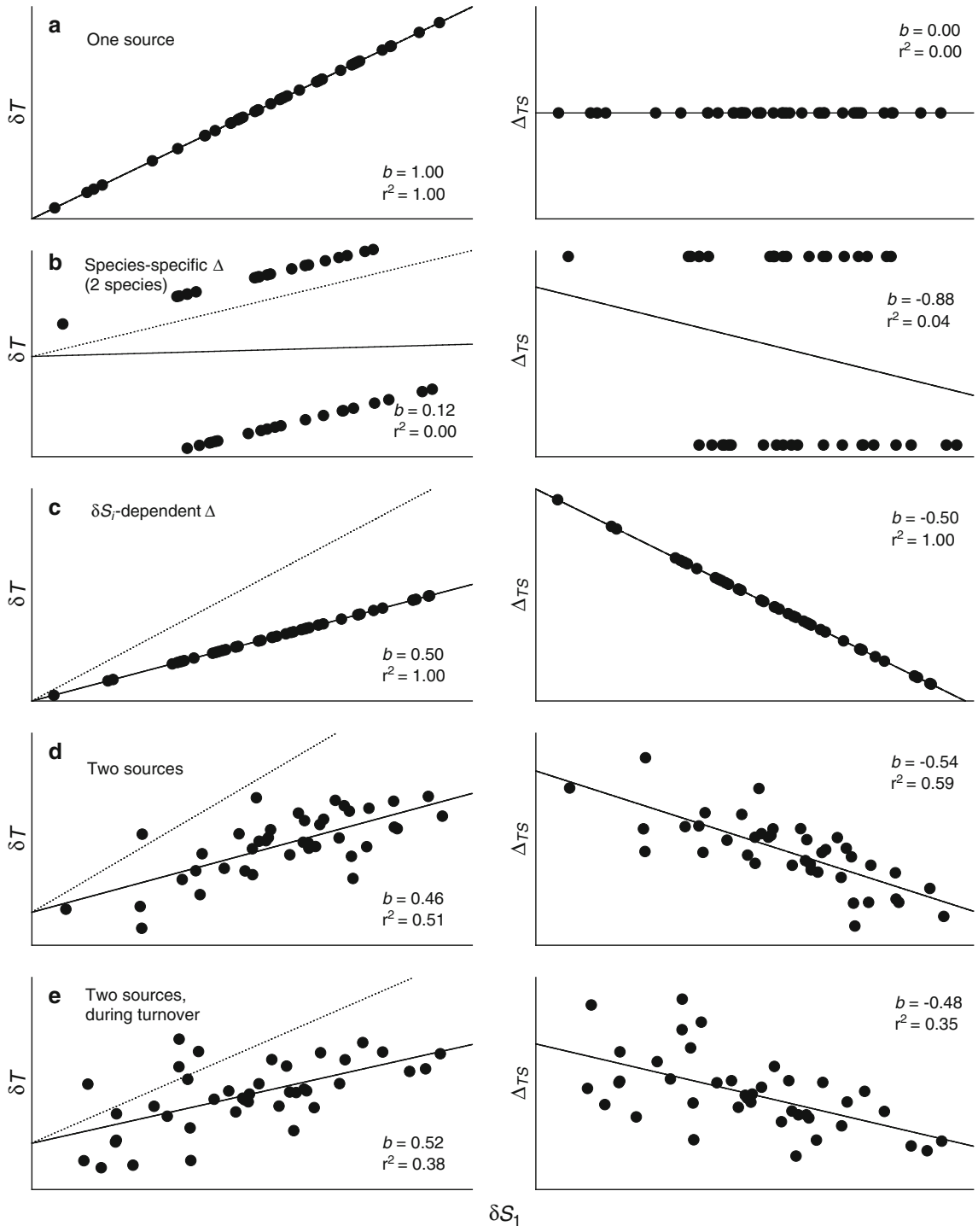

Fig. 1 Simulated hypothetical relationships between stable isotope composition of consumers (δT) and their sources/diets (δS) under different scenarios (*panels on the left*). *Panels on the right* show corresponding relationships between consumer-diet spacing (Δ_{TS}, i.e., $\delta T - \delta S$) and δS. Each scenario corresponds to Eq. 1 through to Eq. 5 in the main text. **a** δT varies with δS of a single source, plus a constant Δ_{TS}. **b**, **c** Changes to Δ_{TS} are assumed to influence the relationship: first due to differences in Δ_{TS} between two species (**b**; two intercepts) and next if Δ_{TS} varies as a negative linear function of δS (**c**). **d**, **e** Relationships assume heterogeneity in δS due to the consumption of different diets or compound-specific differences in δS of a single diet and/or nutrient routing (**d**) and due to non-equilibrium between consumer and diet isotope compositions following a diet switch (**e**). In **d** and **e**, only a single source value is plotted on the *x*-axis, reflecting the common practice of ignoring heterogeneity, such as within laboratory feeds. Note the difference in slopes (**b**) between the single- (**a**–**c**) and multiple-source (**d**, **e**) models. The *solid line* depicts the linear regression, and the *dashed line* depicts a 1:1 relationship, and the increased variation around the regression when multiple sources are included. Axis units and model parameters are not shown as only regression parameters are relevant here

ferences in Δ_{TS} across species using multiple-intercept models like analysis of co-variance (ANCOVA). However, this is an unsatisfying explanation for any arising changes in slope because a separate-slopes model would merely yield a set of slopes = 1.0 for each species.

Caut et al. (2010) provide an alternate hypothesis: that slopes of <1.0 arise because Δ_{TS} is negatively and linearly related to δS. This effect was detected by these authors in two earlier studies of empirical data (Caut et al. 2008, 2009), and this scenario implies that $\Delta_{TS} = a - b\delta S$ (Fig. 1c, right panel). Substituting into Eq. 1 (and ignoring the species-specific scenario in Eq. 2) gives:

$$\delta T = (a - b\delta S) + \delta S \quad (3)$$

where a and b are constants. Equation 3 results in a slope of <1.0 for the relationship between δT and δS (Fig. 1c, left panel). This interpretation has been criticized because it does not consider variations in δT that arise via isotopic routing (Perga and Grey 2010) and because of the spurious correlation between δS and Δ_{TS} that arises because δS appears in the independent and dependent variable (Auerswald et al. 2010). Also, the scenario depicted in Eq. 3 presently has, by its designers' admission, no theoretical or empirical explanation (Caut et al. 2009).

The interpretation presented by Robbins et al. (2010) does entail a mechanistic approach; however, the theory was developed specifically for differences in the Δ_{TS} of the stable isotopes of nitrogen that arise from differences in isotope composition, quality, and digestibility across diet components. We offer here a coarser adaptation of this idea, extending it to all systems in which δS is non-homogeneous. This concept is applicable to isotopes of any element and to any type of resource (food, water, etc.) and provides a simple, yet functional explanation for the reduced slopes of many δT–δS relationships and also accounts for the negative relationship between Δ_{TS} and δS observed by Caut et al. (2009, 2010). If δS is non-homogeneous, this implies that, at any given time, there are multiple isotopically distinct substrates available in the body pool for tissue synthesis. Multiple δS values contribute to δT under several, not necessarily exclusive physical and biological conditions, which have been repeatedly demonstrated:

1. An individual consumes multiple isotopically distinct food types simultaneously or within a relatively short time period; for example, in generalist feeders with mixed diets;
2. Differences arising from the above may be exacerbated if the quality or digestibility of food types differ (as in Robbins et al. 2010; see also Codron et al. 2011);
3. Biochemical components of the diet have compound-specific isotope compositions (even within single feeds), and these components (proteins, lipids, carbohydrates, amino acids) are routed differently to different tissues (Ambrose and Norr 1993; Martínez del Rio et al. 2009).

Under any of the above three conditions, each δS_I value contributes some fraction (f_I) to the value for δT. Summing fractional contributions from n sources gives:

$$\delta T = \sum_{i=1}^{n} \delta S_I f_I + \Delta_{TS}; \sum f_I = 1 \quad (4)$$

Equation 4 forms the basis of linear mixing models, which are widely applied to convert raw isotope data into estimates of ecological niche space (Newsome et al. 2007).

Another source of heterogeneity in δS values arises when the consumer switches to a new diet (S_2) and the δT value is obtained before the tissue is in equilibrium with δS_2 (condition 4). Then, components of the previous diet remain in the body pool and/or have a catabolic origin (Ayliffe et al. 2004), and the function δT (δS_1) is influenced by the time taken for δS_2 to replace δS_1 in the nutrient pool. This "isotope turnover" (Tieszen et al. 1983; Hobson and Clark 1992) follows a negative exponential decay function over time (t)

$$\delta T = \delta S_2 + \Delta_{TS} + (\delta S_1 - \delta S_2)e^{(-\lambda t)} \quad (5)$$

where λ is a rate constant. Equation 5 describes a switch from S_1 to S_2 (note: δS_2 is an asymptotic "equilibrium") and can be modified to accommodate multiple phases of isotope incorporation (Ayliffe et al. 2004; Cerling et al. 2007; Martínez del Rio and Anderson-Sprecher 2008).

It is not our intention to propose mechanistic models here that can differentiate the sources of variation described by the above-mentioned conditions 1 through 4. Rather, we are interested only in statistical phenomena that explain the more general problem of how multiple δS values (occurring across and/or within diets) influence relationships between δT and δS_I. We allowed our 40 hypothetical individuals to each utilize two δS values—first, according to Eq. 4 and, secondly, in non-equilibrium with the new diet (Eq. 5). The result is that slopes for the regression of δT on δS_1 are significantly less than 1.0 (Fig. 1d, e), which occurs because the regression lacks the fraction(s) contributed by the source (S_2) not included on the x-axis (in Fig. 1a, the slope = 1.0 because each individual has a single, i.e., homogeneous δS value, such that $f_I = 1.0$ and is the sole contributor to the δT value). In addition, the contribution of two δS values to each individual means that relatively less of the variation in δT is explained by variation in δS_1, and there is increased variation around the regression (lower r^2). This phenomenon was explicitly noted by Robbins et al. (2010) in their evaluation of empirical datasets. By contrast, our simulations of Caut et al.'s (2008, 2009, 2010) hypothesis do not

predict a change in variance around the regression (Fig. 1c), although increased scatter around regression lines was apparent in their analyses of empirical datasets. Moreover, our multiple source functions generate negative relationships between Δ_{TS} and δS_1 (Fig. 1d, e; panels on the right), but in our simulations the result is an artefact, not a cause as proposed by Caut et al. (2010).

Relationships between consumer tissues

Possibilities other than those shown in Fig. 1 are readily conceivable—for example, differences in food digestibility or source availability could lead to slopes of >1.0 or even nonlinear relationships between δT and δS (e.g., Wittmer et al. 2010; Codron et al. 2011), but more complex hypotheses are beyond the scope of the present study. The simplified concepts outlined here suggest that linear regression parameters of δT (δS) functions can potentially distinguish between effects caused by variations in Δ_{TS} (reduced slopes, but no change in variance explained) from those arising due to isotopic heterogeneity in consumer diets (reduced r^2 values), regardless of whether the latter is due to variation between or within diets.

The same principles should apply to isotopic relationships between consumer tissues because each tissue represents the variation in δS_I values from which it is derived. For example, the isotope compositions of two tissues (δT_1 and δT_2) with similar growth and turnover rates and similar biochemical compositions should be related as in Fig. 1a (i.e., with slope and r^2 not different from 1.0). If, however, one (Fig. 2a) or both (Fig. 2b) tissues incorporate isotopes from multiple substrates (multiple δS values; see Eq. 4), but in different proportions—e.g., because of routing or because diet isotope compositions differ during the period of formation of each tissue—the relationship resembles Fig. 1d (i.e., with slope and $r^2 < 1.0$). Similarly, if the two tissues differ in metabolic and thus isotope turnover rates (different λ in Eq. 5) and are not in isotopic equilibrium with δS_2, the relationship between them also reflects multiple δS contributions (Fig. 2c). Actually, in these cases it is conceivable that slopes of >1.0 could occur, for example if the less heterogeneous tissue was plotted on the *y*-axis, but variance around the regression line will always remain high. Importantly, though, relationships between tissues also mirror consumer-diet relationships of Fig. 1 in that relationships between Δ_{T2-T1} and δT_1 have negative slopes in all systems influenced by multiple δS values (Fig. 2, panels on the right).

Here, we test these predictions based on isotopic relationships between gut contents, gut tissue, hair, and bone collagen of South African savanna herbivores. If our assertions are accurate, we expect that linear models will reveal r^2 and slopes approaching 1.0 for materials derived from similar source mixtures—for example, between ingesta sampled from different sections of the gastrointestinal tract (assuming no influence of changes in carbon:nitrogen composition along the tract) and between proteinaceous body tissues. However, because gut contents represent short-term bulk intake while proteinaceous body tissues are synthesized from dietary proteins and are integrated over longer periods, relationships between gut contents with body tissues should reflect their construction from different sources, i.e., with r^2 and slopes that are substantially less than 1.0.

Materials and methods

The sample for this study comprised seven species of large mammal herbivores from two reserves (Soetdoring and Tussen-die-Riviere Nature Reserves) situated in the grassland biome of the central interior of South Africa. The habitat for herbivores in this region is a homogeneous, open landscape, with mostly high grass productivity and little or no tree cover (Rutherford and Westfall 1994). Six of the species sampled are ruminants, namely, the greater kudu *Tragelaphus strepsiceros* (Pallas, 1766) ($n = 10$), the springbok *Antidorcas marsupialis* (Zimmerman, 1780) ($n = 10$), the oryx *Oryx gazella* (L., 1758) ($n = 5$), the blue wildebeest *Connochaetes taurinus* (Zimmerman, 1780) ($n = 8$), the blesbok *Damaliscus pygargus phillipsi* (Pallas, 1767) ($n = 6$), and the red hartebeest *Alcelaphus buselaphus* (Pallas, 1766) ($n = 2$), and one species is a hindgut fermenter [common warthog *Phacochoerus africanus* (Gmelin, 1788), $n = 10$]. Based on field observations and previous stable carbon isotope studies, these taxa can be classified across three trophic guilds: browser (kudu), intermediate-feeder (springbok), and grazers (Skinner and Smithers 1990; Gagnon and Chew 2000; Sponheimer et al. 2003a; Codron et al. 2007).

Animals were shot during routine hunting programs of the Free State Nature Conservation in 2007. At Tussen-die-Riviere NR, tissue collections were made in the field, within 30 min postmortem, but nighttime visibility at Soetdoring NR was poor and so the entire gut contents were retained in cool storage and sampled the following morning. From each individual, our aim was to sample gut contents (rumen, or forestomach in the case of warthog), reticulum content (mostly fluid; ruminants only), gut wall (rumen or stomach lining; carbon isotope data for this tissue in ruminants are from Codron and Clauss 2010), hair, and bone. Gut contents were sampled as handfuls, but the entire contents of the reticulum were extracted and mixed, and a subsample was used for analysis. Hair was collected in clumps, including proximal and distal

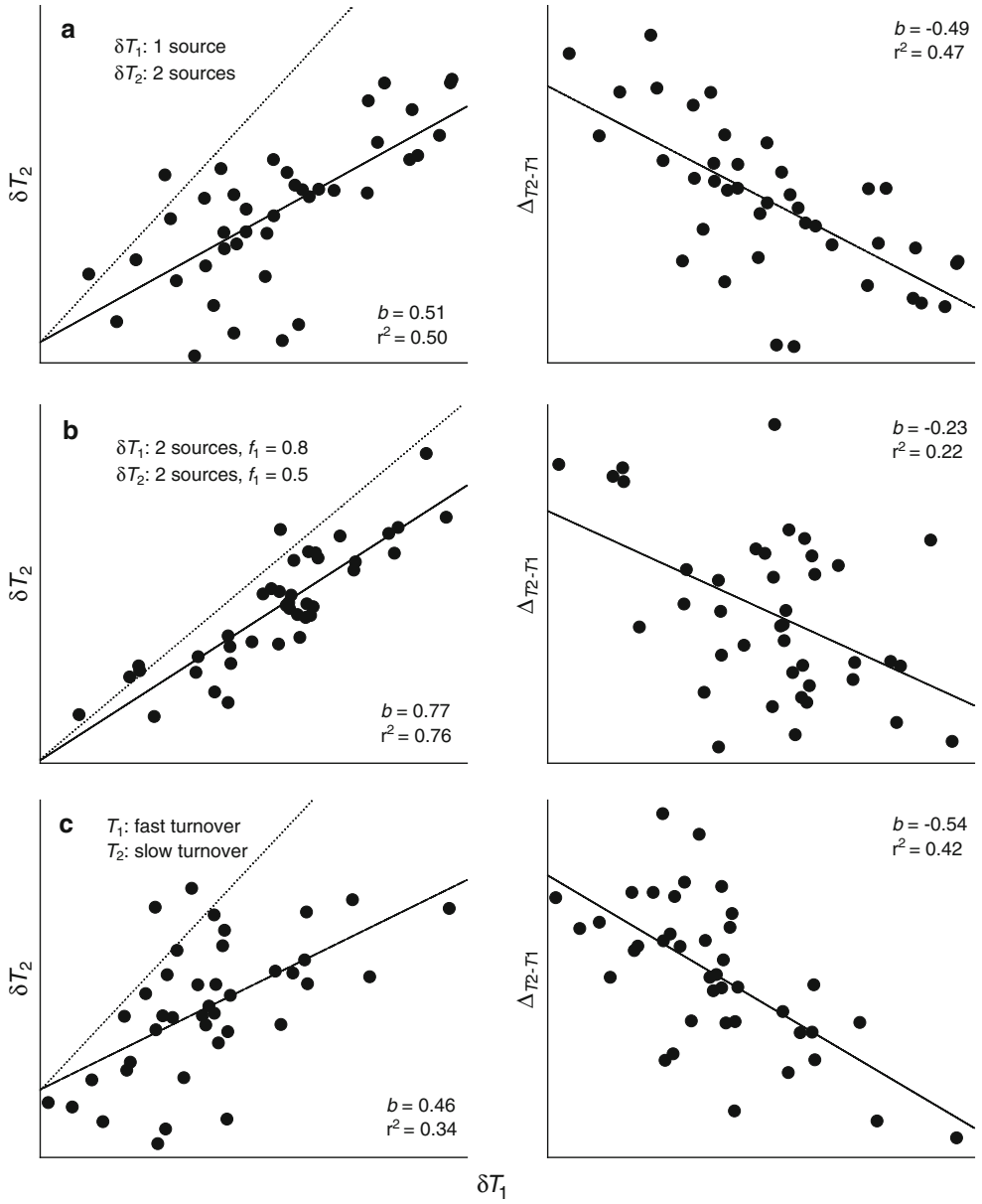

Fig. 2 Relationship of stable isotope compositions of two consumer tissues (T_1 and T_2) based on similar concepts used to produce Fig. 1. In all cases, we assumed heterogeneity in δS values, i.e., δS_1 and δS_2, contributing to either tissue in different proportions (**a**, and f_I in **b**) or being incorporated at different rates (**c**). *Panels on the right* show the corresponding negative relationships that arise between dT1 and the spacing (Δ) between δT_2 and δT_1

parts, to randomize the growth phase represented. Bone fragments were removed from mandibles with pliers.

All materials were stored frozen until laboratory analysis. In the laboratory, thawed samples were rinsed with distilled water and freeze-dried overnight at −40°C for isotope analysis. Bone fragments were treated for isolation of the protein (collagen) phase in 0.2 M HCl, and lipids were removed by treatment in a methanol:chloroform:water solution. All materials were analyzed for $^{13}C/^{12}C$ and $^{15}N/^{14}N$ composition of organic compounds by stable light isotope mass spectrometry, following methods reviewed elsewhere (e.g., Codron et al. 2007). The results are reported using the delta (δ) notation, relative to Vienna PeeDee Belemnite (VPDB) and atmospheric N_2 standards, respectively. Analytical precision for these analyses, i.e., standard deviations for laboratory standards, was better than 0.2‰.

We calculated isotopic spacing between each tissue and gut contents using the latter material as baseline because it is the closest we have to the actual diet ingested. We used

the scale-independent model of isotopic enrichment (ε, in units ‰) between two components of a reaction following Craig (1954):

$$\varepsilon_{\text{material}-\text{gutcontents}} = \left(\frac{10^3 + \delta X_{\text{material}}}{10^3 + \delta X_{\text{gutcontents}}} - 1\right) 10^3 \quad (6)$$

This calibration is preferred above the arithmetic difference (Δ) because it provides a more accurate estimation across a wide range of δ-values (Cerling and Harris 1999; Passey et al. 2005). We present estimates of ε with subscripts R, W, H, B, and G to indicate reticular fluid, gut wall, hair, bone collagen, and gut contents, respectively, and ε_{TG} to indicate the offset between any consumer material and G.

Data analysis

Initially, we compared the data between species and the materials analyzed using repeated measures (RM) ANOVA, with "material" and "species" as within-subjects and between-subjects factors, respectively. Dependent variables were δ and ε_{TG} values. Four RM ANOVA models were used, depending on the availability of data for each species: for example, warthog does not have a reticulum, thus models with reticular fluid as a material excluded this species. Similarly, bone collagen and hair samples were unavailable for oryx. Significance levels were set at 0.05 and, where necessary, multiple comparisons were investigated using Bonferonni post hoc tests.

Relationships between materials were evaluated by simple linear regressions, initially with δ_G as the independent variable and δ values for all other materials as dependent variables. The relationships between body tissues were then evaluated by testing regressions of δ_B and δ_H on δ_W, and finally of δ_B on δ_H. For all models, a random error term was introduced to both variables, drawn from a normal distribution with a mean of 0 and variance ±1.0. Regression parameters and 95% confidence intervals (CIs) were computed by bootstrapping (10^3 iterations) and compared against our predictions outlined above. Similarly, we evaluated regressions of ε_{TG} on δG for slopes deviating from 0.

Analyses were carried out using STATISTICA Enterprise v8.0 for RM ANOVAs (Statsoft_Inc 2007) and PopTools v3.0.6 (Hood 2008) for bootstrap iterations of regressions. Although raw δ^{13}C data are bimodal, residuals were always normally distributed and had equal variances. In addition, predictions shown in Fig. 1 were identical when available δS values were assumed to have a bimodal (i.e., C_3/C_4) distribution [see Electronic Supplementary Material (ESM)].

Results

Differences across tissues and species

There are significant effects of species and material type, as well as an interaction effect, on both δ^{13}C and δ^{15}N (RM ANOVA $p < 0.01$ in all cases). Multiple comparisons revealed consistent patterns throughout, despite data for some materials being absent for certain species.

δ^{13}C values for all materials from kudu were significantly lower (by up to 12‰) than values for the same materials from other taxa (Fig. 3a). These data are consistent with a C_3-dominated diet, as expected for browsers. δ^{13}C values for all grazer species were consistent with C_4-dominated diets, and no individual data points for this group overlapped with the range for kudu. Amongst grazers, warthog had significantly higher (approx. 2‰) δ^{13}C values for gut contents but lower δ^{13}C for body tissues (1–2‰) than grazing ruminants. δ^{13}C values for materials from springbok were intermediate between those of kudu and grazers, reflecting the mixed browse/grass diet of this species. δ^{13}C for springbok body tissues did not overlap with the range observed for kudu and for grazers, but three individuals had values for reticular fluid that were similarly enriched in ^{13}C compared with oryx and wildebeest.

Similarly, there was a significant effect of species on ε_{TG} for δ^{13}C ($p < 0.0001$), but only because springbok and warthog had smaller offsets than the other species (Fig. 3b). No significant differences in ε_{TG} for δ^{13}C were found between the other taxa for any material, including between browsing (kudu) and grazing ruminants ($p > 0.47$).

There were also differences in δ^{13}C values between materials, which were up to 6‰ within species and even within some individuals, ranked as follows: gut contents < reticular fluid < gut wall < hair < bone collagen ($p < 0.05$ for all comparisons; Fig. 3a). ε_{TG} varied in like fashion, i.e. reticular fluid < gut wall < hair < bone collagen ($p < 0.01$ for all comparisons; Fig. 3b).

Differences in mean δ^{15}N across species implied three apparent contrasts. The lowest values were found for warthog, and the highest (up to 14‰ greater than warthog) were found for springbok, oryx, and blesbok (Fig. 3c). Kudu, wildebeest, and hartebeest had intermediate δ^{15}N values. Species effects on ε_{TG} of ^{15}N showed the inverse trend (Fig. 3d). In other words, taxa with the highest mean δ^{15}N (springbok, blesbok, and oryx) had lower ε_{TG} than other ruminants ($p < 0.05$), and warthog—which had lowest mean δ^{15}N—had highest ε_{TG} amongst all species ($p < 0.05$).

There were also differences in δ^{15}N across materials, but these were inconsistent along the species axis. In kudu, warthog, wildebeest, and hartebeest, the lowest values were found for gut contents, then reticular fluid, and the highest values were found for gut wall ($p < 0.05$ in all cases),

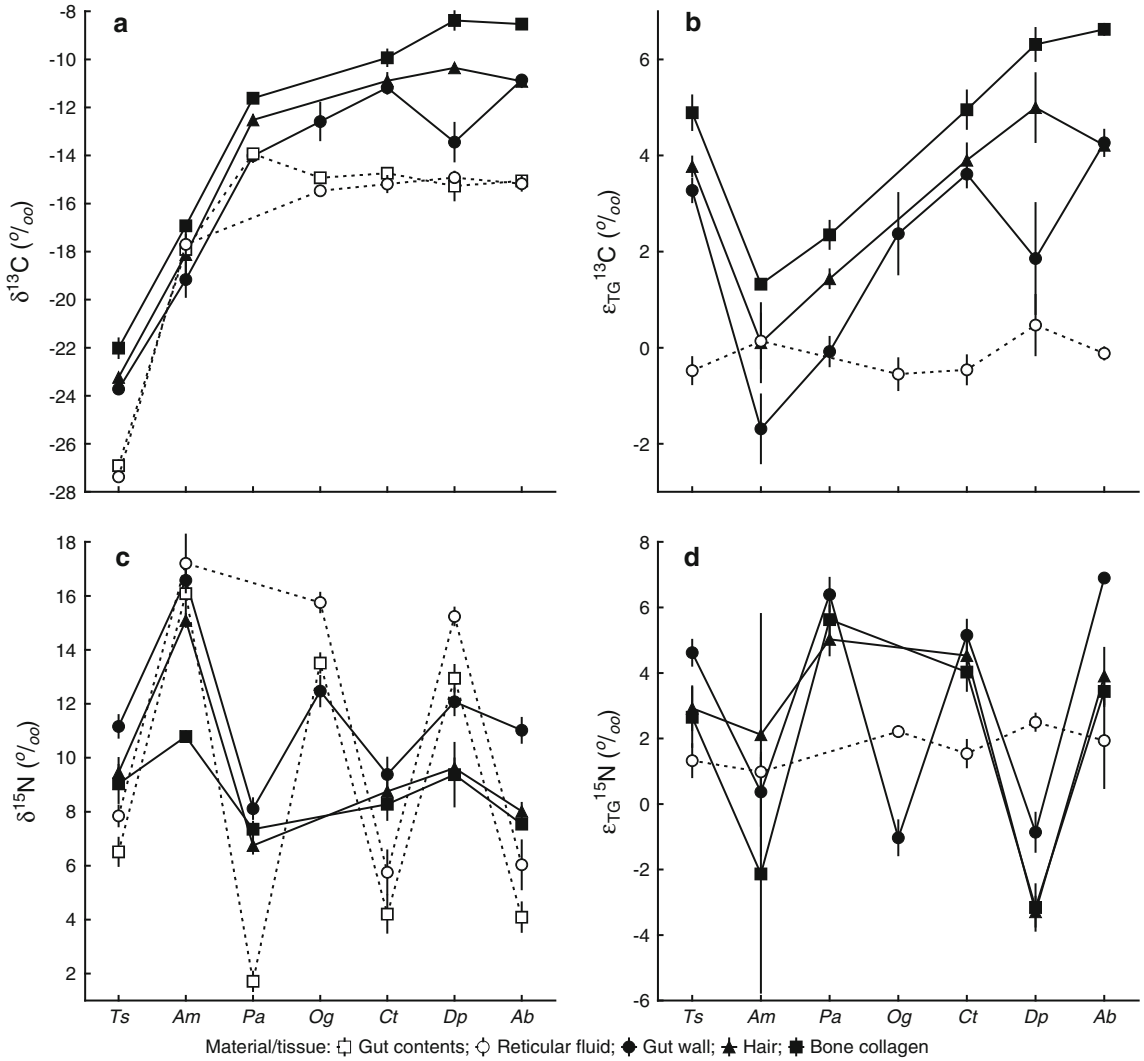

Fig. 3 Comparisons of mean δ values, and enrichment relative to gut contents (ε_{TG}), of multiple materials/body tissues across the seven herbivore species in this study. **a**, **b** Data for $\delta^{13}C$, **c**, **d** data for $\delta^{15}N$. *Symbols* depict means ± 1 standard error of material/tissue analyzed. The *lines* track interspecific trends for each material/tissue type. Species: *Ts* Greater kudu (*Tragelaphus strepsiceros*), *Am* springbok (*Antidorcas marsupialis*), *Pa* warthog (*Phacochoerus africanus*), *Og* oryx (*Oryx gazella*), *Ct* blue wildebeest (*Connochaetes taurinus*), *Dp* blesbok (*Damaliscus pygargus phillipsi*), *Ab* red hartebeest (*Alcelaphus buselaphus*).

whereas gut contents of springbok, oryx, and blesbok had $\delta^{15}N$ values only slightly higher, or even similar to, that of the gut wall (Fig. 3c). Hair and bone collagen $\delta^{15}N$ values were, in most taxa, intermediate between that of gut contents and gut wall, but hair and bone $\delta^{15}N$ values did not differ from each other ($p > 0.15$). Variations in ε_{TG} paralleled these patterns, being lowest (and not different from zero) for reticular fluid, highest in gut wall, and intermediate and similar for hair and bone collagen, but again there were inconsistent trends across species (Fig. 3d).

Stable isotope relationships

Linear regression models revealed significant relationships between all materials for both $\delta^{13}C$ and $\delta^{15}N$ ($p < 0.0001$), which varied in strength according to the nature of the material, with some species-level effects. The relationship between $\delta^{13}C$ values of ingesta in different components of the gastrointestinal tract (rumen content and reticular fluid) had a slope not different from 1.0 and high r^2 (0.89) (Table 1; Fig. 4a). Relationships between $\delta^{13}C$ values of gut contents with body tissues yielded slopes significantly less than 1.0, with more scatter around the regression lines (Fig. 4b) and hence lower r^2 (0.71–0.85), whereas between body tissues, slopes again included 1.0 at 95% confidence (Fig. 4c). The deviation in slopes away from 1.0 observed in gut content–body tissue relationships was mainly because two taxa (springbok and warthog) fell consistently below the 1:1 line. Indeed, the omission of these species from the analysis resulted in slopes that were

Table 1 Parameter estimates for simple linear regression models of isotopic relationships between all materials and body tissues sampled, including analyses of a subset excluding the two species (springbok and warthog) for which regressions often had slopes of <1.0 ($\delta^{13}C$ only)

Condition	Material/tissue type	n	r^2	Intercept	Slope	Slope for ε_{TG} on δ_{GC}
$\delta^{13}C$ (all taxa included)						
Between gut contents	RF on GC	38	0.89 (0.84–0.93)[a]	−0.88 (−2.43 to 0.84)	0.96 (0.88–1.05)	0.00 (−0.06 to 0.06)
Between gut contents and body tissues	GW on GC	49	0.71 (0.63–0.77)	−1.52 (−2.96 to −0.06)	0.82 (0.75–0.90)	−0.14 (−0.21 to −0.08)
	H on GC	38	0.85 (0.80–0.90)	1.35 (−0.07 to 2.75)	0.90 (0.82–0.97)	−0.08 (−0.14 to −0.01)
	B on GC	30	0.80 (0.72–0.87)	2.20 (0.64–3.98)	0.89 (0.80–1.00)	−0.08 (−0.15 to 0.00)
Between body tissues	H on GW	38	0.87 (0.81–0.91)	0.80 (−0.54 to 2.19)	0.98 (0.90–1.06)	
	B on GW	30	0.84 (0.77–0.90)	2.36 (0.65–4.29)	1.00 (0.90–1.12)	
	B on H	30	0.90 (0.84–0.94)	0.95 (−0.55 to 2.49)	0.98 (0.88–1.01)	
$\delta^{13}C$ (springbok and warthog excluded)						
Between gut contents	RF on GC	30	0.92 (0.88–0.96)	−0.71 (−2.38 to 1.05)	0.98 (0.90–1.07)	0.00 (−0.06 to 0.07)
Between gut contents and body tissues	GW on GC	31	0.87 (0.82–0.92)	1.55 (−0.12 to 3.26)	0.93 (0.85–1.01)	−0.05 (−0.11 to 0.02)
	H on GC	26	0.92 (0.87–0.95)	4.13 (2.29–6.19)	1.00 (0.92–1.10)	0.03 (−0.04 to 0.09)
	B on GC	18	0.93 (0.87–0.97)	6.07 (3.85–8.42)	1.04 (0.93–1.16)	0.06 (−0.02 to 0.15)
Between body tissues	H on GW	26	0.89 (0.83–0.93)	0.75 (−0.88 to 2.46)	0.99 (0.89–1.09)	
	B on GW	18	0.87 (0.80–0.93)	2.94 (0.91–5.07)	1.04 (0.92–1.17)	
	B on H	18	0.93 (0.88–0.97)	1.76 (0.04–3.68)	1.02 (0.91–1.14)	
$\Delta^{15}N$ (all taxa included)						
Between gut contents	RF on GC	38	0.87 (0.82–0.91)	2.28 (1.31–3.15)	0.93 (0.84–1.02)	−0.04 (−0.10 to 0.03)
Between gut contents and body tissues	GW on GC	49	0.65 (0.56–0.74)	7.75 (7.19–8.26)	0.45 (0.39–0.50)	−0.52 (−0.58 to −0.47)
	H on GC	38	0.34 (0.21–0.47)	6.95 (6.36–7.52)	0.32 (0.24–0.39)	−0.63 (−0.72 to −0.55)
	B on GC	30	0.29 (0.13–0.47)	7.12 (6.54–7.69)	0.23 (0.15–0.31)	−0.72 (−0.83 to −0.63)
Between body tissues	H on GW	38	0.36 (0.21–0.52)	2.73 (0.80–4.42)	0.59 (0.43–0.77)	
	B on GW	30	0.26 (0.10–0.45)	4.53 (2.81–6.20)	0.38 (0.22–0.56)	
	B on H	30	0.33 (0.16–0.53)	4.71 (3.28–6.13)	0.42 (0.27–0.58)	

GC, Gut contents; RF, reticular fluid; GW, gut wall; H, hair; B, bone collagen, ε_{TG}, enrichment relative to gut contents

[a] Values presented in parenthesis are the 95% confidence intervals (95% CIs); these limits around the means are derived from 10^3 bootstrap iterations, with error terms introduced for both x- and y-axis variables

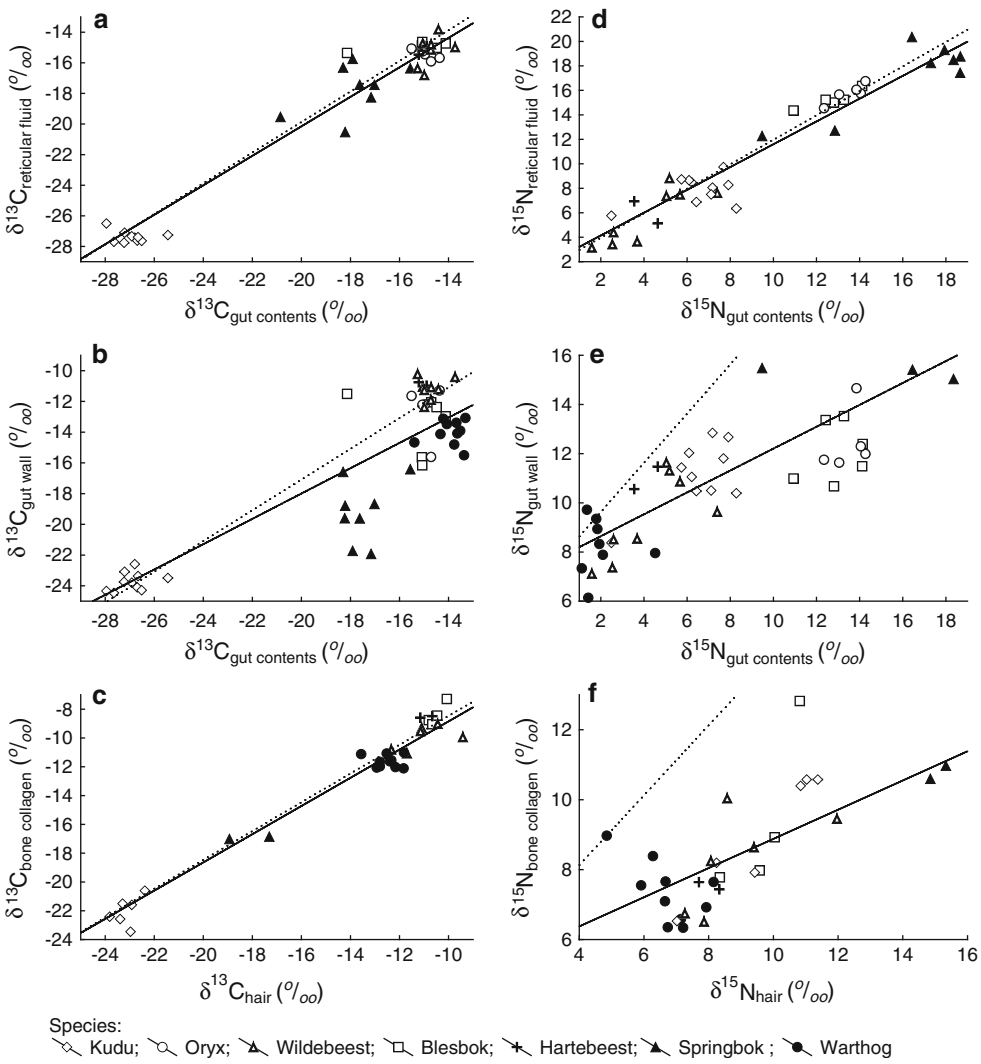

Fig. 4 Some $\delta^{13}C$ (**a**–**c**) and $\delta^{15}N$ (**d**–**f**) relationships between materials/tissue types included in this study. *Solid lines* represent linear regressions through the data, *dashed lines* represent a model with the same intercept, but with slope = 1.0. **a**, **d** Comparison of two components of gastrointestinal tract contents, **b**, **e** comparison of gut contents with a proteinaceous body tissue, **c**, **f** comparison of two proteinaceous body tissues. Note the influence of the mixed-feeder (springbok) and hindgut fermenter (warthog) (both in *black symbols*) on the regression in **b**. Details of regression parameters for relationships between all materials/tissue types are provided in Table 1

not significantly different from 1.0, and regressions with high r^2 (0.87–0.93; Table 1).

For $\delta^{15}N$, the relationship between gut contents with reticular fluid again yielded a slope not different from 1.0 (Table 1; Fig. 4d). As with $\delta^{13}C$, relationships between $\delta^{15}N$ of body tissues and gut content had slopes substantially smaller than 1.0 and reduced r^2 (0.29–0.65; Fig. 4e). However, unlike $\delta^{13}C$ data, the relationships in $\delta^{15}N$ between proteinaceous body tissues also had slopes of <1.0 (Fig. 4f), and no outlier taxon (like springbok or warthog above) was consistently discernable.

Consistent with theoretical predictions, regressions of ε_{TG} on δG yielded slopes significantly different from 0 (negative) only in cases where the relationship between δ values of the various materials had slopes of less than 1.0 (Table 1).

Discussion

These results demonstrate tissue- and species-specific isotope signatures, some of which can be explained by the effects of isotopic heterogeneity across or within diets. We first discuss these effects before addressing the more general problem of failure to address them.

Effects of mixed diets on tissue–tissue relationships

We proposed that relationships between the stable isotope compositions of various tissues in individuals can be interpreted in the same way as relationships between stable isotope compositions of animal tissues and their diets. The latter, which already have a robust theoretical and empirical background, are influenced by changes in the level of isotopic heterogeneity in the diet (different diets or compound-specific differences within diets) and/or by changes in isotope fractionation effects for different species or diets (DeNiro 1978, 1981; Cerling and Harris 1999), as exemplified in Fig. 1. We tested predictions for similar effects in multiple tissue analyses of free-ranging mammalian herbivores, but with the limitation that we used data for gut contents as isotopic baselines because the isotopic

composition of free-ranging herbivore diets is not known. Our results are consistent with the effects of different levels of source isotopic heterogeneity, with fractionation changing only as an artefact of this.

For $\delta^{13}C$, the relationships between contents from different regions in the gut, as well as relationships between different proteinaceous body tissues, had near-perfect linear slopes (not different from 1.0). However, relationships between gut contents and body tissues had slopes of <1.0 and greater variation around the regression. Our initial interpretation is that the carbon in the contents of both components of the digestive tract are derived from the same dietary source and, similarly, that carbon in the gut wall, hair, and bone collagen of these animals is derived from the same source or combination of sources. This result was expected. First, ruminants mix rumen and reticulum contents repeatedly during digestion, especially during rumination; second, all body tissues we analyzed are protein-based and hence derived from similar components of the body nutrient pool (Ambrose and Norr 1993). By contrast, the carbon in the gut contents and body tissues are likely derived from a dissimilar combination of sources, linked to the time between ingestion and (later) production of body tissues.

Our interpretation of a heterogeneous signal in gut content–body tissue relationships is supported by the fact that deviations from linearity were caused by the two species for which isotopically heterogeneous diets are the most likely (springbok and warthog). The springbok is one of few African herbivores that habitually switches between browsing and grazing, usually between dry and wet seasons, or which consumes both food types simultaneously (Skinner and Smithers 1990; Gagnon and Chew 2000). Gut contents of springbok were sampled in the late wet season (March), a time when fresh grass is most abundant in these habitats, and when mixed-feeders are most likely to eat more grass (du Toit 2003). Not surprisingly, $\delta^{13}C$ values of springbok gut contents were more similar to—or overlapping with—values for grazers than browsers (kudu). However, springbok body tissues had $\delta^{13}C$ values intermediate between values for grazers and the browser, which should be expected if these materials represent a more mixed diet signal integrated over a longer time period. Warthog, as suids, could be partly omnivorous, but even as strict herbivores they are more likely to consume a wider variety of foods than many grazing ruminants, for example by digging for roots and bulbs, some of which may be C_3 (Skinner and Smithers 1990). Therefore, warthogs are also more likely to have body tissues reflecting a variety of dietary sources rather than the fresh grass found in their guts. Additionally, a specific fractionation arising via differences in digestive physiology cannot be ruled out for this species; warthog are hindgut fermenters, whereas all other taxa in our sample are ruminants. A proper test for this effect should reveal separate intercepts (Fig. 1b), but requires more hindgut fermenter species.

Excluding springbok and warthog, the remaining species in our sample are stenotopic browsers or grazers. In this subset, even the gut content-body tissue $\delta^{13}C$ relationships had slopes not different from 1.0. In other words, the detection of temporally heterogeneous diets was lost when mixed-feeders were excluded. We do not imply that these browsing and grazing ruminants do not vary their diets within their respective feeding niches, such as by switching seasonally between plant species and plant parts (Skinner and Smithers 1990; du Toit 2003), but simply that such switches do not entail much carbon isotope heterogeneity across or within resources. Mixed source signals could have been found had we sampled gut contents over different seasons, as shown from carbon isotope analysis of browser and grazer feces (Codron et al. 2007).

For $\delta^{15}N$, the relationships again suggest that rumen and reticulum contents share common N sources, whereas gut contents and body tissues are assimilated from dissimilar combinations. However, relationships between proteinaceous tissues were weak with gentle slopes (Table 1), suggesting a greater temporal variability in source $\delta^{15}N$, which could account for the wide variations in data across species. Another possible explanation is that dietary proteins are broken down into their constituents, from which amino acid chains are reassembled only during tissue synthesis. Fractionation effects during synthesis should result in compound-specific ^{15}N compositions across amino acids, and this heterogeneity is manifest as differences across body proteins according to their amino acid profiles (see Martínez del Rio et al. 2009). Accordingly, although different body tissues may have similar $\delta^{15}N$ (from the same bulk body pool), their respective $\delta^{15}N$ values are only partially—or even only incidentally—correlated.

The dilemma in these data, however, is not so much the mismatch in relationships between tissues as the large differences in $\delta^{15}N$ across species (Fig. 3c). Large differences between gut contents could occur through post-mortem protein degradation and/or microbial blooms, especially because a proliferation of microbes could lead to shifts in $\delta^{15}N$ values in a positive or negative direction depending on the substrate (Wattiaux and Reed 1995). Yet, if this were occurring, we might expect much weaker relationships between rumen and reticular content than those observed here. Regardless, large interspecific differences were found within each body tissue type as well, for which postmortem effects can hardly be implicated. For some tissues, differences across species were as large as 8–10‰, levels which would in some systems be consistent with shifts of two to five trophic steps (Post 2002).

Actually, the level of $\delta^{15}N$ variation we observed here is extraordinarily high compared to that normally observed in large mammal herbivore systems, including that from studies conducted over sub-continental scales (Sealy et al. 1987; Murphy and Bowman 2006). Interspecific trends in our data do not correspond to differences in diet (browser, grazer, or mixed-feeder), digestive physiology (ruminant or hindgut fermenter), phylogenetic affiliation, geographical origin, sampling protocol, nor trophic level. Similar inconsistencies (of smaller magnitude) have been found across herbivore species on controlled diets (Sponheimer et al. 2003b). For the present, added caution is probably necessary for many interpretations of ecological patterns from nitrogen stable isotopes.

Prospects for isotope analysis of gut contents

The carbon isotope results presented here demonstrate that, in these habitats, analysis of a wide variety of herbivore materials can be used to differentiate between browsing, grazing, and intermediate feeding. Diet differentiation on this scale has been shown repeatedly from the analysis of feces, hair, bone collagen, tooth dentine collagen, tooth enamel carbonate, and other tissues (Vogel 1978; Tieszen et al. 1979; Cerling and Harris 1999; Sponheimer et al. 2003a; Codron et al. 2007). Gut contents are a valuable addition to this list (see also Tieszen et al. 1979).

Because (fore-) gut contents should be largely consistent with food intake, analysis of these may provide elusive information about the magnitude of ε_{TS} in natural settings. These values are normally obtained from controlled-feeding studies, and field researchers must assume similar values for the same or related species (Martínez del Rio et al. 2009; Newsome et al. 2010). For many taxa, such as large mammals, long-lived turtles, or endangered species, experimental studies to generate values for ε_{TS} are impractical, since they must often be conducted over long time periods to ensure isotopic equilibrium between consumers and a homogeneous experimental diet. Also, assuming constant ε_{TS} from laboratory to field situations is often inappropriate because climatic variations, diet, and other stress factors outside the laboratory have effects (Newsome et al. 2010, and references therein).

For many herbivores, ε_{TG} may be equal or related to ε_{TS}. Excluding the springbok and warthog, our use of gut contents as a dietary baseline yielded mean offsets to bone collagen [5.4‰ ± 0.25 standard error (SE)] and hair (4.1‰ ± 0.22 SE) that are comparable with tissue-diet spacings obtained from laboratory and field experiments (approx. 4.0–6.0‰ and 3.1–3.9‰, respectively) (Ambrose and Norr 1993; Cerling and Harris 1999; Wittmer et al. 2010). These results and the linear slopes of isotopic relationships further imply that little or no differences in ε_{TG} (or ε_{TS}) occurred across ruminant species with different diets—despite the many morphological and physiological traits that differentiate browsing from grazing ruminants (Clauss et al. 2008) and despite differences in the digestibility of C_3 browse and C_4 grass (Heckathorn et al. 1999).

When mixed-source signals are present in relationships between gut contents and body tissues, they should trace digestive processes in free-ranging animals. In ruminants, grass is retained for longer time periods in the rumen, increasing exposure to bacterial fermentation processes necessary for the digestion of fiber-rich forage, whereas mean retention time for browse is shorter (Hummel et al. 2006; Lechner et al. 2010; Clauss et al. 2011). The longer retention of grass in the rumen could thus partially explain the higher-than-expected $\delta^{13}C$ values of springbok gut contents, while the lower-than-expected $\delta^{13}C$ values of springbok body proteins could reflect a situation in which relatively more metabolic proteins are derived from the C_3 browse component of the diet, even when C_4 grass consumption rates are high (see Codron et al. 2011). Concurrent analysis of gut contents and body tissues at seasonal scales could also reveal specific shifts in digestive pathways if isotopic changes are prevalent further along the digestive tract (Hwang et al. 2007; Codron et al. 2012).

Back to basics: the meaning of individual-level relationships

Stable isotope relationships between tissues and between consumers and their diets are informative about diet composition, and the magnitude of fractionation effects (spacing). However, researchers need to be aware of the factors that cause deviations in these relationships to avoid misinterpreting patterns. It is important to note that parameters and interpretations derived from relationships between source isotope signatures (δS) and spacing (Δ_{TS}, or ε_{TS}) (Caut et al. 2008, 2009; Robbins et al. 2010) are likely to be in error because δS appears in both axes, leading to a spurious correlation (Auerswald et al. 2010). Robbins et al. (2010) argued that the parameters of the δT (δS) function are biased by an autocorrelation, because "the *x*-axis is diet and the *y*-axis is diet plus discrimination". We believe this is not the case: discrimination (tissue-diet isotope spacing) is an abstraction, not an empirical measure, whereas both δT and δS are independent empirical measures (in different materials) that are strongly related, making stable isotope approaches to diet possible.

To set up predictions for this study, we employed a set of very simple (and not novel) hypothetical scenarios. Our approach is in broad agreement with that of Robbins et al. (2010), and further cautions that researchers pay special attention to the effects of isotopic heterogeneity between—and within—(food) sources. The conditions associated with

these scenarios (e.g., delayed isotope turnover, compound-specific isotope heterogeneity, routing) are well-known. Actually, most multiple tissue studies are primed by these effects, i.e., that differences reflect differences in source contributions and can therefore be used to measure extent and/or timing of diet switching (Hobson 1999; Phillips and Eldridge 2006). Isotope-based models of ecological niche have already shown how source heterogeneity across space and time can influence consumer signatures and confound data interpretation (Matthews and Mazunder 2004; Codron et al. 2007; Flaherty and Ben-David 2010). How deviations in consumer-diet isotope relationships influence niche models is crucial for advancing these approaches.

A more pressing immediate concern is that effects of diet source heterogeneity are entirely overlooked in many systems, especially in data from controlled experiments where single diets are assumed to be isotopically homogeneous. For example, an alternative interpretation of our dataset could have been that relationships are regulated by effects of dietary δ values on tissue-diet spacing (see Fig. 1c), the so-called "Diet-Dependent Discrimination Factor", or DDDF (Caut et al. 2009). Despite lacking a functional explanation, Caut et al. (2009) "strongly recommend" applying DDDFs to all isotope studies of wildlife. However, basic theory shows such relationships to be numerical artefacts (Fig. 1d, e; see also Auerswald et al. 2010). Simulations of Caut et al.'s (2009, 2010) original interpretation revealed that this would lead to no variance around the regression ($r^2 = 1.0$; Fig. 1c), whereas models assuming isotopic heterogeneity in sources (Fig. 1d, e) produced large variance around regressions. The latter are more consistent with Caut et al.'s (2009, 2010) observations (r^2 between approx. 0.05 and 0.53).

In our data, a functional DDDF should have resulted in (1) no reductions in r^2 when slopes were <1.0 and (2) slopes <1.0 persisting even when taxa with mixed diets (springbok) were omitted. Evidence from a controlled-feeding study (Codron et al. 2011) is consistent with our argument: in that case there was no difference in ε_{TS} of ^{13}C between animals on C_3 and C_4 diets, and hence the negative relationship between tissue-diet spacing and diet δ-values also disappeared when mixed diets were excluded from the analysis (see also Wittmer et al. 2010). In their investigations, Caut et al. (2008, 2009) most likely detected effects because of isotopic heterogeneity in the diet isotope signal (Perga and Grey 2010). Heterogeneity could also have arisen in experiments in which consumer tissues were not in perfect equilibrium with diet (although the authors attempted to omit such data where possible), or if fractions of experimental feeds varied in quality, digestibility, and compound-specific isotope composition (Martínez del Rio et al. 2009; Robbins et al. 2010). The latter should be considered the rule, not the exception, for many diets including compound feeds, such as pelleted and other common laboratory diets. There may be another explanation for trends reported in Caut et al. (2008, 2009), but until one is provided we "strongly recommend" that researchers avoid the use of patterns which arise from assumptions of isotopic homogeneity within sources—like the DDDF. Rather, parameters of consumer-diet and within-consumer isotopic relationships can be exploited for differentiating patterns of source heterogeneity (both between and within diets) in the field and in the laboratory.

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